

Evaluation of Antioxidant and Anti-Inflammatory Effect of *Rhododendron brachycarpum* Extract Used in Skin Care Product by *in Vitro* and *in Vivo* Test

Amal Kumar Ghimeray, Ha Youn Lee, Young Hoon Kim, Eun Kyung Ryu, Moon Sik Chang*

R&D Center, Natural Solution Co., Ltd., Incheon, South Korea

Email: justin0510@naturalsolution.co.kr, amal_ghimeray@yahoo.com

Received 9 January 2015; accepted 25 April 2015; published 28 April 2015

Copyright © 2015 by authors and Scientific Research Publishing Inc.

This work is licensed under the Creative Commons Attribution International License (CC BY).

<http://creativecommons.org/licenses/by/4.0/>



Open Access

Abstract

In this study, we aimed to observe the cytotoxicity, antioxidant and anti-inflammatory activity of *Rhododendron brachycarpum* extract (RbE) in RAW 264.7 macrophage cell *in vitro* and the anti-inflammatory or soothing effect of RbE formulated cream (Multicalm-RH™) on experimentally induced acute irritated skin in healthy subjects *in vivo*. *In vitro* study revealed that RbE significantly inhibited NO and ROS generation in a dose dependent manner without cytotoxicity. In clinical test, Multicalm-RH™ cream significantly reduced the SLS-induced erythema after D5 compared to that of placebo. Overall, it can be concluded that the RbE is a non-cytotoxic, antioxidant and anti-irritant agent which can be used as a potent functional ingredient in cosmetics. Likewise, the *in vivo* clinical study shows that Multicalm-RH™ cream which contains 3% RbE can be used as an effective skin soothing agent.

Keywords

Antioxidant, Anti-Inflammatory, Cytotoxic, *Rhododendron brachycarpum*, Cosmetics

1. Introduction

Environmental exposure (UV radiation, pollutions, toxic chemicals and other hazardous agents) causes contact dermatitis, halogen acne, chemical depigmentation, skin cancer and other acute and chronic skin damages [1].

*Corresponding author.

Recently, to overcome this problem, the cosmetic industries and academic researchers have devoted much research toward the development of various natural skin care product from plant parts.

Plants are the natural source of functional ingredients. Phytochemicals (secondary metabolites) from the plants are characterized for having antioxidant, anti-inflammatory, anti-carcinogenic, anti-aging and anti-bacterial properties [2]. The effective plants antioxidant compounds such as tocopherols, flavonoids, phenolic acids, alkaloids, monoterpenes are widely used in cosmetic products to protect the skin from environmental damages [3].

Rhododendron brachycarpum G. Don belongs to the family Ericaceae which has been used in folk medicine for the treatment of diabetes, arthrities, headache and hypertension [4] [5]. The plant is reported to have antimicrobial and antioxidant activity [6]. Similarly, the plant also possesses anti-inflammatory, anti-tyrosinase and moisturizing effect [7] [8]. This plant consists of important flavonoid compounds, in particular, quercetin, avicularin, quercetrin and hyperin [9]. Previously, antioxidant and anti-inflammatory activity of *Rhododendron brachycarpum* extract (RbE) has been researched [10], however, no evidences of clinical effectiveness have been published. Recently, cosmetic company (Natural Solution Co. Ltd., South Korea) is using RbE as an active ingredients in soothing skin care products (Multicalm-RH™). Therefore, to examine the efficacy of the extract and its product, we aimed to observe the cytotoxicity, antioxidant and anti-inflammatory activity in RAW 264.7 macrophage cell *in vitro* and the soothing effect of RbE containing cream (Multicalm-RH™) on experimentally induced acute irritated skin in healthy subjects *in vivo*.

2. Materials and Methods

2.1. RbE Sample Preparation

Rhododendron brachycarpum G. Don (leaf and stem) was collected from the forest of Taebaek (650 m - 700 m from sea level), Kangwon-Do province, South Korea. The samples were dried in oven at 45°C for 5 days and ground to a fine powder. The powdered sample was extracted with 40% 1, 3-propanediol by ultrasonic extraction method for 3 days. The extracts were filtered and concentrated by evaporation under freeze-dried. The condensed sample was used in the experiment after dilution.

2.2. Soothing Agent

MultiCalm-RH™ cream (Natural Solution Co. Ltd., South Korea) containing the following ingredients were used in the experiment: *Rhododendron brachycarpum* extract (3%) mixed with Water, Glycerine, Disodium EDTA (ethylene diamine tetra-acetic acid), Methylparaben, Carbomer, Tocopheryl acetate, Polysorbate 60, Triethanolamine, Stearyl alcohol, PEG-100 (polyethylene glycol-100) stearate, Sorbitan stearate, caprylic/capric triglyceride, Dimethicone, Mineral oil, Propylparaben, Butylene Glycol, Beeswax, and Fragrance. The placebo control was identical in composition, except for RbE.

2.3. Cell Culture, Cytotoxicity and Anti-Inflammatory (Nitrite) Determination *in Vitro*

Murine macrophage cell line RAW 264.7 was purchased from the Korea cell line bank, Seoul, Korea were cultured in 96-well plates containing Dulbecco's Modified Eagle Medium (DMEM, 200 µl/well) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin sulfate (100 µg/ml) in a humidified atmosphere of 5% CO₂. The cells (2×10^5 /well) were incubated with RbE at different concentrations (0.5%, 1% and 2%) in the presence of 1 µg/ml lipopolysaccharide (LPS) for 24 hours. After overnight incubation, aliquots of 100 µl of cell culture media was mixed with 100 µl gress reagent (50 µl of 1% Sulfamylamide in 5% phosphoric acid and 50 µl of 0.1% Naphthylethylenediamine-HCl), and incubated at room temperature for 10 min. Absorbance at 550 nm was determined using an ELISA reader (SpectraMax M5, Molecular devices). The concentration of nitrite was determined by comparison with sodium nitrite standard curve, as reported previously [11]. The cell cytotoxicity assay of the RbE was performed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent following the protocol of Mosmann *et al.* [12].

2.4. Evaluation of Antioxidant (ROS Inhibition) Activity *in Vitro*

Antioxidant was evaluated in reactive oxygen species (ROS) inhibition model according to the protocol of Wang *et al.* [13] with minor modification. Briefly, 1×10^4 cells/well were plated into 96-well plates 1 day before the

experiments. On the day of the experiments, after removing the medium, different concentrations (0.5%, 1% and 2%) of sample were loaded and incubated for 1 hour in 5% CO₂/95% air at 37°C for 30 min. After removing the medium, the cells in the plates were washed with PBS buffer and treated with 20 mM dichlorofluorescein diacetate (DCFH-DA) and 1 mM H₂O₂ (free radical generator) for 90 min. ROS levels were measured time-dependently (at intervals of 10 min) with an excitation wavelength of 485 nm and emission wavelength of 530 nm using fluorescence spectrophotometer.

2.5. Anti-Inflammatory (Soothing Effect) Clinical Test *in Vivo*

40 subjects (30 women and 10 men) aged 18 - 65 years were participated in the research were provided written informed consent. None of the participant had the history of atopy dermatitis, allergies, any skin related diseases, pregnancy, any systemic and topical medication during the last 2 months. The study was a randomized, placebo-controlled which was approved by Guang Dong Light Industry Association Institutional Review Board [14]. 3% Sodium lauryl sulfate (SLS) was used as an irritant following the protocol of Tupker *et al.* [15]. During the experiment, the patch testing procedure was same as reported previously in our other experiment [14]. Briefly, large Finn chambers (+12 mm) with filter disc were fixed to mark test sites on the flexor side of fore arm in the subjects. On D0, normal skin was irritated with occlusive application of 3% SLS on flexor sides of both forearms for 24 hours. The next day (D1), Six hours after removing the patches, approximately 2 mg·cm⁻² (40 mg) of cream or placebo was applied to the test site (treated twice a day) for 24 hours for 9 consecutive days. Erythema index was measured using Mexameter MX18 (Cologne, Germany) on D1 (before treatment), D3, D5, D7 and D9. The visual erythema scores were evaluated by dermatologist using the inflammatory severity which contains 10 scales: “0” indicated no erythema, whereas “9” indicated severe erythema. During the investigation, the room temperature and humidity was maintained at 22°C ± 1°C and 50% ± 3% respectively.

2.6. Statistical Analysis

Statistical analyses were carried out using SPSS software (version 11.5; SPSS Inc., Chicago, IL, USA). The differences among samples were statistically evaluated via one-way analysis of variance (ANOVA) followed by Dunnett’s posthoc test or Wilcoxon’s test when appropriate. The level of significance was set at $P < 0.05$. Data are expressed as means ± standard errors.

3. Results

3.1. Determination of Anti-Inflammatory, Antioxidant and Cytotoxicity Effect of RbE *in Vitro*

The anti-inflammatory effect of RbE tested on macrophage RAW 264.7 is shown in **Figure 1**. The result revealed that the RbE dose-dependently inhibited LPS-induced NO production. At the concentration of 0.5%, 1% and 2%, RbE inhibited NO up to 5.48%, 13.73% and 21.82% respectively. In ROS inhibition activity, RbE also inhibited in a dose-dependent manner. At the concentration of 1% and 2%, RbE inhibited ROS upto 17.09% and 26.42% respectively. Due to the significant ($P < 0.05$) inhibition activity of RbE in dose dependent manner on the NO and ROS generation, the cytoprotective effect of extract on LPS-induced cytotoxicity was investigated (**Figure 2**). According to the data, the extract did not induce cytotoxicity up to 2% concentration used in the experiment. These results suggested that the inhibition of ROS and NO production by RbE might be managed by radical scavenging activity.

3.2. Erythema Index and Visual Score

In this study, occlusive application of 3% SLS for 24 hours in the test fields resulted in a strong increase of skin erythema. Application of the cream (MultiCalm-RH) onto the test skin on D1 did not show any significant difference ($P < 0.05$) with placebo. However, the significant ($P < 0.05$) reduction of the erythema index compared with placebo were observed on D5 (**Figure 3**). The visual erythema scores of the sides treated by cream decreased after D3 (**Figure 4**). On D9, the visual erythema score of the cream was 2.46 lower than that of the placebo, and the erythema value of the cream was 49.75 lower than that of the placebo.

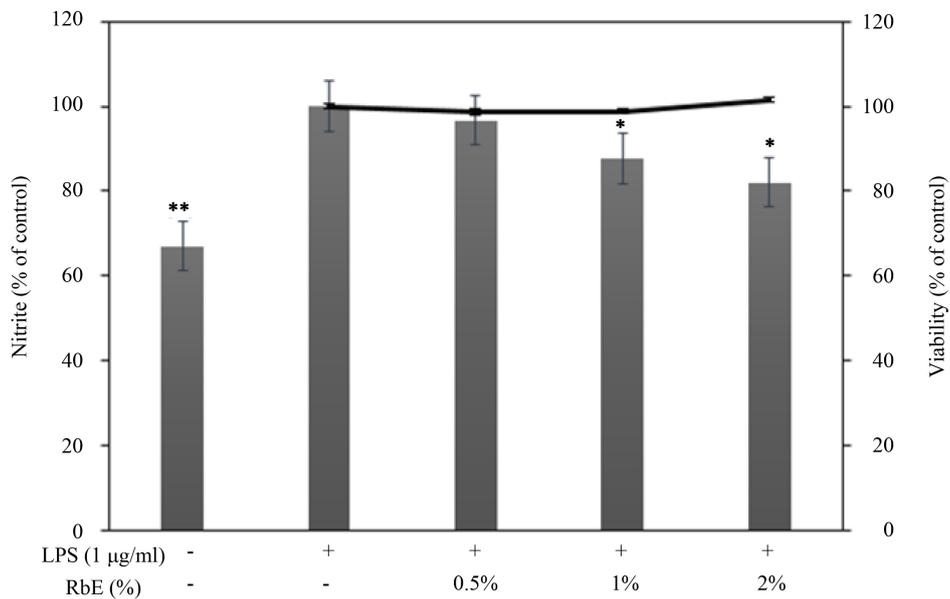


Figure 1. The cell viability was determined by the amount of MTT converted to the insoluble formazan salt after the treatment with different concentration of RbE in Macrophage cell. The effect of RbE on the production of NO in lipopolysaccharide-induced RAW 264.7 macrophage cells was measured by using Griess reagent. The data are representative of three different experiments with similar results. * $P < 0.05$, ** $P < 0.01$, vs control (LPS-treated, no sample treatment). NO: nitric oxide, LPS: lipopolysaccharide, RbE: *Rhododendron brachycarpum* extract.

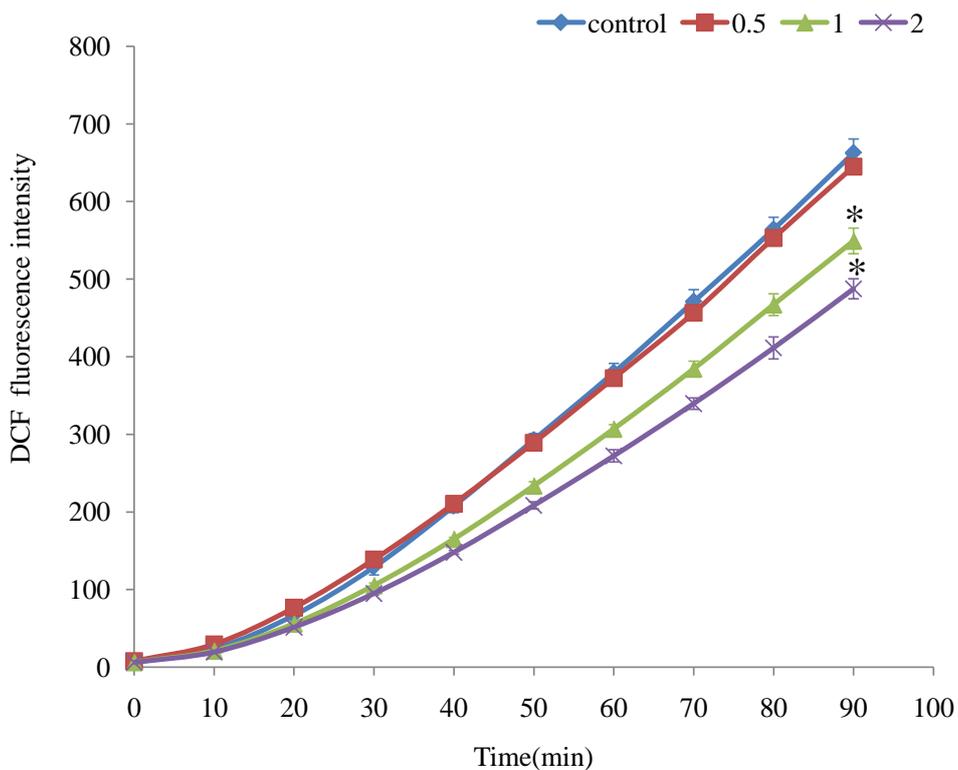


Figure 2. Effect of *Rhododendron brachycarpum* extract on ROS generation inhibition activity in RAW 264.7 macrophage cells. The data are representative of three different experiments with similar results. Asterisk (*) indicates significance at the $P < 0.05$ level compared to control.

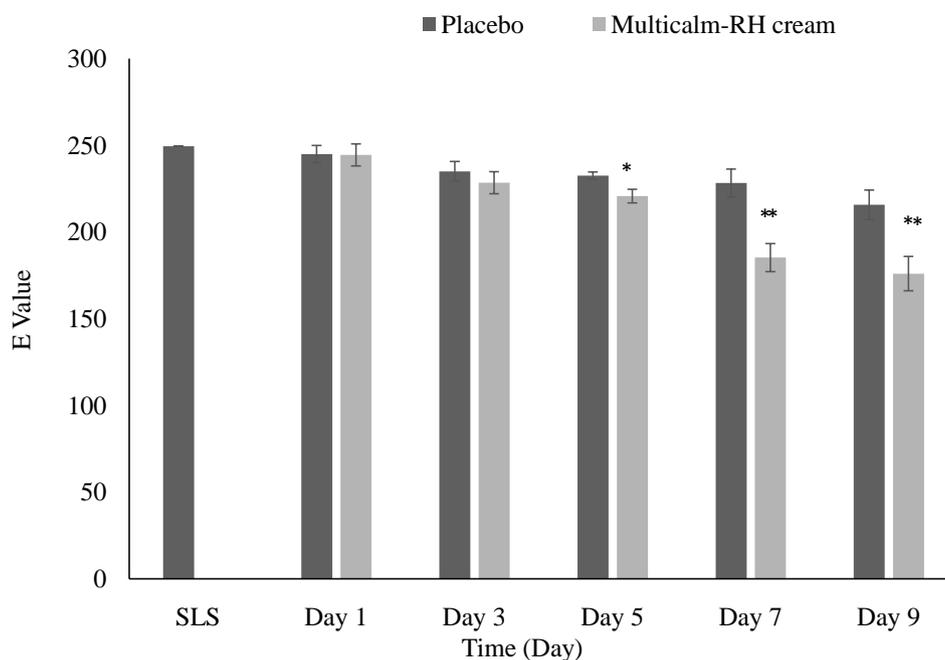


Figure 3. Differences in the Erythema value after the treatment of Multicalm-RHTM cream or the placebo randomly on SLS-induced skin irritation of healthy subjects. Data are expressed as mean \pm SD. * $P < 0.05$, ** $P < 0.01$ compared to that of placebo.

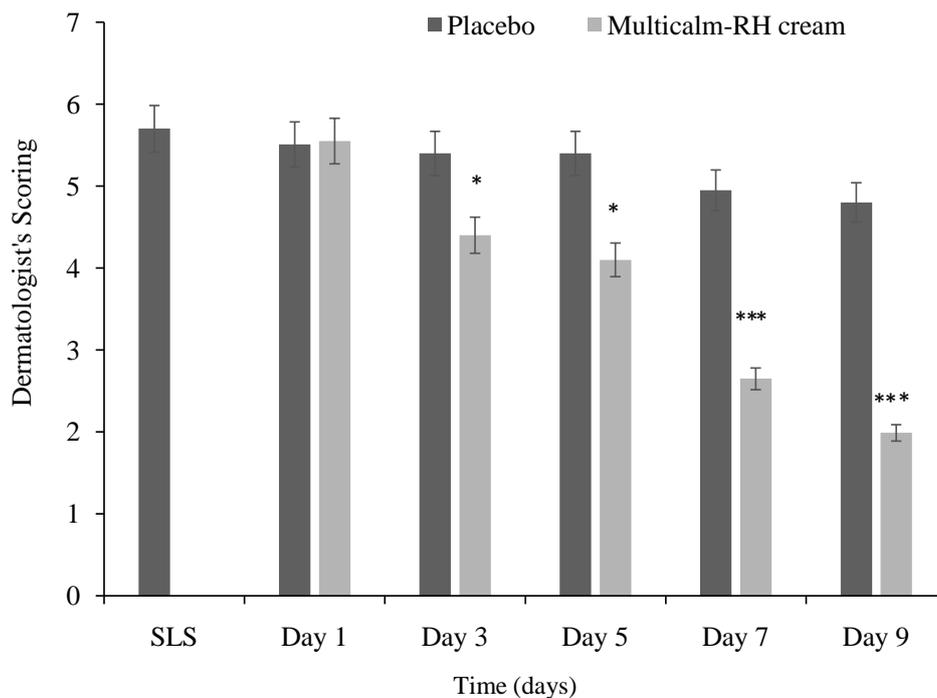


Figure 4. Differences in the visual score after the treatment of Multicalm-RHTM cream or the placebo randomly on SLS-induced skin irritation of healthy subjects. Data are expressed as mean \pm SD. * $P < 0.05$, *** $P < 0.001$ compared to that of placebo.

4. Discussion

It has been reported that ROS plays an important role in an acute skin inflammation [16]. To reduce the inflam-

mation, application of moisturizers or ointment often assists in the improvement of irritant contact dermatitis [17]. Recently, there is an increasing scientific interest of exploring plant ingredients as anti-inflammatory or soothing agents. *Rhododendron barchycarpum* plant extract (RbE) has been used as a soothing agent in skin care products. In our study, RbE inhibits LPS-induced NO production in dose-dependent manner. At the concentration of 2% dose, RbE inhibits NO production significantly with 26.42% compared with control. The dose dependent inhibition of NO by RbE can be due to the presence of bioactive compound like Rhododendrin in the extract. In previous research [10], the Rhododendrin isolated from Rb plant showed strong anti-inflammatory activity by inhibiting LPS induced NO production significantly ($P < 0.05$) and also inhibited iNOS protein and mRNA expression in a concentration dependent manner. According to our data in antioxidant assay, RbE inhibited ROS in dose dependent manner in dichlorofluorescein assay system. In cytotoxic assay, the extract does not induce cytotoxicity up to 2% of concentration used in the experiment. These all observations indicate that RbE is a significant antioxidant and anti-inflammatory agent without toxic effect *in vitro*.

According to new regulations for cosmetics, manufacturers of skin care products have to prove the efficacy of the product [18]. An authentic data in a short time and easy application on the forearms of healthy subjects are highly desirable [19]. Therefore, we evaluate the effects of MultiCalm-RHTM cream on forearms of 40 subjects to evaluate the anti-inflammatory efficacy *in vivo*. In cosmetics, sodium lauryl sulphate (SLS) is most popular model for studying skin irritation and skin barrier damage. It has been reported that the SLS can damages the skin without systematic toxicity or carcinogenic properties [17]. Therefore, we have investigated the anti-inflammatory effect of cream in the SLS test to facilitate the penetration of creams through the epidermal skin barrier. In this study, 3% SLS is used to induce severe visual inflammation. Otherwise, weaker concentration is known to cause disturbance of barrier function with a single application [20]. The resulting irritation is quantified by a visual score or by photometric measurement of the erythema using a Mexameter. The study is designed as a 9-day application, because we have no preliminary information on how long it will take for the skin barrier to start repairing under test condition. The visual erythema scores and the erythema value of cream treatment are significantly ($P < 0.05$) lower than those of placebo after D5. This result suggests that the cream can reduce the skin inflammation which shows its anti-inflammatory effect *in vivo*.

Overall, it can be concluded that the RbE is a non-cytotoxic, antioxidant and anti-irritant agent which can be used as a potent functional ingredient in cosmetics. Likewise, the *in vivo* clinical study shows that Multicalm-RHTM cream which contains 3% RbE can be used as an effective skin soothing agent.

Acknowledgements

The authors gratefully acknowledge to Skin Research Center of Guangzhou Land proof and Department of Dermatology, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong province, China, for conducting the *in vivo* research work.

Conflict of Interest

There are no conflicts of interest.

References

- [1] English, J.S.C., Dawe, R.S. and Ferguson, J. (2003) Environmental Effects and Skin Disease. *British Medical Bulletin*, **68**, 129-142. <http://dx.doi.org/10.1093/bmb/ldg026>
- [2] Middleton, E. and Kandaswami, C. (1986) The Impact of Plant Flavonoids in Mammalian Biology: Implications for Immunity, Inflammation and Cancer. In: Harbone, J.B., Ed., *The Flavonoids: Advances in Research Since 1986*, Chapman & Hall, London, 619-652.
- [3] Korać, R.R. and Kapil, M.K. (2011) Potential of Herbs in Skin Protection from Ultraviolet Radiation. *Pharmacognosy Reviews*, **5**, 164-173. <http://dx.doi.org/10.4103/0973-7847.91114>
- [4] Choi, Y.H., Zhou, W., Oh, J., Choe, S., Kim, D.W., Lee, S.H. and Na, M. (2012) Rhododendric Acid A, a New Ursane-Type PTP1B Inhibitor from the Endangered Plant *Rhododendron brachycarpum* G. Don. *Bioorganic & Medicinal Chemistry Letters*, **22**, 6116-6119. <http://dx.doi.org/10.1016/j.bmcl.2012.08.029>
- [5] Zhou, W., Oh, J., Li, W., Kim, D.W., Yang, M.H., Jang, J.H., Ahn, J.S., Lee, S.H. and Na, M. (2014) Chemical Constituents of the Korean Endangered Species *Rhododendron brachycarpum*. *Biochemical Systematics and Ecology*, **56**, 231-236. <http://dx.doi.org/10.1016/j.bse.2014.06.003>

- [6] Popescu, R. and Brigitte, K. (2013) The Genus *Rhododendron*: An Ethnopharmacological and Toxicological Review. *Journal of Ethnopharmacology*, **147**, 42-62. <http://dx.doi.org/10.1016/j.jep.2013.02.022>
- [7] Zhou, W., Oh, J., Wonhwa, L., Kwak, S., Li, W., Chittiboyina, A.G., Ferreira, D., Hamann, M.T., Lee, S.H., Bae, J.S. and Na, M. (2014) The First Cyclomegastigmane Rhododendroside A from *Rhododendron brachycarpum* Alleviates HMGB1-Induced Sepsis. *Biochimica et Biophysica Acta*, **1840**, 2042-2049. <http://dx.doi.org/10.1016/j.bbagen.2014.02.016>
- [8] Park, J.O., Gyu, N.L. and Su, N.P. (2010) Antioxidant Activity of *Rhododendron brachycarpum* D. Don Extracts and Its Skin Hydration Effect Measure. *Journal of Society Cosmetic Scientists of Korea*, **36**, 157-165.
- [9] Choi, J.S., Young H.S., Park, J.H., Choi, J.H. and Woo, W.S. (1986) Flavonoids from the Leaves of *Rhododendron brachycarpum*. *Archives of Pharmacal Research*, **4**, 233-236. <http://dx.doi.org/10.1007/BF02856638>
- [10] Jeon, Y.J., Kim, B.H., Kim, S., Oh, I., Lee, S., Shin, J. and Kim, T.Y. (2013) Rhododendrin Ameliorates Skin Inflammation through Inhibition of NF- κ B, MAPK, and PI3K/Akt Signaling. *European Journal of Pharmacology*, **714**, 7-14. <http://dx.doi.org/10.1016/j.ejphar.2013.05.041>
- [11] Isabelle, B. and Hiroshi, O. (1995) Curcumin, an Anti-Tumour Promoter and Anti-Inflammatory Agent, Inhibits Induction of Nitric Oxide Synthase in Activated Macrophages. *Biochemical and Biophysical Research Communications*, **206**, 533-540. <http://dx.doi.org/10.1016/j.bbrc.2013.05.041>
- [12] Mosmann, T. (1983) Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods*, **65**, 55-63. [http://dx.doi.org/10.1016/0022-1759\(83\)90303-4](http://dx.doi.org/10.1016/0022-1759(83)90303-4)
- [13] Wang, H. and Joseph, J.A. (1999) Quantifying Cellular Oxidative Stress by Dichlorofluorescein Assay Using Microplate Reader. *Free Radical Biology & Medicine*, **27**, 612-616. [http://dx.doi.org/10.1016/S0891-5849\(99\)00107-0](http://dx.doi.org/10.1016/S0891-5849(99)00107-0)
- [14] Zhang, Y.Q., Guan, L., Zhong, Z.Y., Chang, M., Zhang, D.K., Li, H. and Lai, W. (2014) The Anti-Inflammatory Effect of Cherry Blossom Extract (*Prunus yedoensis*) Used in Soothing Skin Care Product. *International Journal of Cosmetic Science*, **36**, 527-530. <http://dx.doi.org/10.1111/ics.12149>
- [15] Tupker, R.A., Willis, C., Berardesca, E., Lee, C.H., Fartasch, M., Agner, T. and Serup, J. (1997) Guidelines on Sodium Lauryl Sulfate (SLS) Exposure Tests. A Report from the Standardization Group of the European Society of Contact Dermatitis. *Contact Dermatitis*, **36**, 53-69. <http://dx.doi.org/10.1111/j.1600-0536.1997.tb00041.x>
- [16] Wilhelm, K.P., Freitag, G. and Wolff, H.H. (1994) Surfactant-Induced Skin Irritation and Skin Repair. *Journal of the American Academy of Dermatology*, **30**, 944-949. [http://dx.doi.org/10.1016/S0190-9622\(94\)70114-8](http://dx.doi.org/10.1016/S0190-9622(94)70114-8)
- [17] Katsarou, A., Davoy, E., Xenos, K., Armenaka, M. and Theoharides, T.C. (2000) Effect of an Antioxidant (Quercetin) on Sodium-Lauryl-Sulfate-Induced Skin Irritation. *Contact Dermatitis*, **42**, 85-89. <http://dx.doi.org/10.1034/j.1600-0536.2000.042002085.x>
- [18] Serup, J. (1998) Legal Situation and Study of Efficacy and Tolerability of Topical Products on Human Skin Using Non-Invasive Methods. *Current Problems in Dermatology*, **26**, 203-208. <http://dx.doi.org/10.1159/000060563>
- [19] Wigger-Alberti, W., Caduff, L., Burg, G. and Elsner, P. (1999) Experimentally Induced Chronic Irritant Contact Dermatitis to Evaluate the Efficacy of Protective Creams *In Vivo*. *Journal of the American Academy of Dermatology*, **40**, 590-596. [http://dx.doi.org/10.1016/S0190-9622\(99\)70442-1](http://dx.doi.org/10.1016/S0190-9622(99)70442-1)
- [20] Lee, J.Y., Effendy, I. and Maibach, H.I. (1997) Acute Irritation Contact Dermatitis Recovery Time in Man. *Contact Dermatitis*, **36**, 285-290. <http://dx.doi.org/10.1111/j.1600-0536.1997.tb00002.x>